Conclusions—These results suggest that SDG reduced hypercholesterolemic atherosclerosis and that this effect was oxidative stress parameters and on the development of atherosclerosis in rabbits fed a high-cholesterol diet. Method and Results—Rabbits were assigned to 4 groups: group 1, control; group 2, SDG control (15 mg·kg body wt−1·d−1 PO); group 3, 1% cholesterol diet; and group 4, same as group 3 but with added SDG (15 mg·kg body wt−1·d−1 PO). Blood samples were collected before (time 0) and after 4 and 8 weeks of experimental diets for measurement of serum triglycerides, total cholesterol (TC), and LDL, HDL, and VLDL cholesterol (LDL-C, HDL-C, and VLDL-C). The aorta was removed at the end of the protocol for assessment of atherosclerotic plaques; malondialdehyde, an aortic tissue lipid peroxidation product; and aortic tissue chemiluminescence, a marker for antioxidant reserve. Serum TC, LDL-C, and the ratios LDL-C/HDL-C and TC/HDL-C increased in groups 3 and 4 compared with time 0, the increase being smaller in group 4 than in group 3. Serum HDL-C decreased in group 3 and increased in group 4 compared with time 0, but changes were lower in group 3 than in group 4. SDG reduced TC and LDL-C by 33% and 35%, respectively, at week 8 but increased HDL-C significantly, by >140%, as early as week 4. It also decreased TC/LDL-C and LDL-C/HDL-C ratios by ~64%. There was an increase in aortic malondialdehyde and chemiluminescence in group 3, and they were lower in group 4 than in group 3. SDG reduced hypercholesterolemic atherosclerosis by 73%.

Conclusions—These results suggest that SDG reduced hypercholesterolemic atherosclerosis and that this effect was associated with a decrease in serum cholesterol, LDL-C, and lipid peroxidation product and an increase in HDL-C and antioxidant reserve. (Circulation. 1999;99:1355-1362.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ flaxseed ■ chemiluminescence ■ antioxidants

Oxygen free radicals (OFRs) have been implicated in the development of hypercholesterolemic atherosclerosis. Polymorphonuclear leukocytes (PMNLs) seem to be one of the sources of OFRs during hypercholesterolemia. Hypercholesterolemia could increase the release of platelet-activating factor (PAF), which in turn could increase the synthesis and release of interleukin-1 and tumor necrosis factor. Leukotriene B4, PAF, interleukin-1, and tumor necrosis factors are known to stimulate PMNLs to produce OFRs, which would initiate the development of hypercholesterolemic atherosclerosis.

Flaxseed contains 35% of its mass as oil, of which 55% is α-linolenic acid (ω-3 fatty acid) and 15% to 18% is linoleic acid. It is a rich source of ω-3 fatty acids and the richest source of plant lignans. Flaxseed has been shown to be effective in reducing hypercholesterolemic atherosclerosis by 46% without lowering serum cholesterol. Crop Development Center (CDC)-flaxseed, which has an oil content (35% of total mass) and concentrations of lignan secoisolariciresinol diglucoside (SDG) (16.4 mg/g versus 15.4 mg/g defatted meal) similar to those of ordinary flaxseed but has only 2% to 3% of α-linolenic acid content, reduced the development of hypercholesterolemic atherosclerosis by 69% and reduced serum cholesterol and LDL cholesterol (LDL-C) by ~31% to 32%. These results suggest that the antiatherogenic activity of flaxseed is not due to α-linolenic acid but may be due to the lignan component of the meal.

Recently, methods have been developed by Agriculture and Agri-Food Canada, a member of our “Flaxseed-Lignan Research Consortium,” to produce SDG (>95% pure) in large quantities (Westcott and Muir). The level of SDG in flaxseed varies between 0.6 and 1.8 g/100 g. The chemical structure of SDG is shown in Figure 1. Its molecular weight is 686.71. Because of its anti-PAF activity, SDG would...
inhibit PAF-induced release of OFRs by PMNLs, and because of its antioxidant activity, it would remove OFRs produced by cells in the body. Hypercholesterolemic atherosclerosis is associated with an increase in the lipid peroxidation product malondialdehyde (MDA), an index of levels of OFRs, and a decrease in antioxidant reserve of the aorta. Reduction in atherosclerosis by antioxidants was associated with a decrease in MDA and an increase in antioxidant reserve of the aorta. It is hypothesized that SDG, which has anti-PAF and antioxidant activity, would prevent development of hypercholesterolemic atherosclerosis. An investigation was therefore made of the effects of a high-cholesterol diet in rabbits with or without SDG treatment on the genesis of atherosclerosis, serum lipid profile [triglycerides (TG), total cholesterol (TC), HDL cholesterol (HDL-C), LDL-C, VLDL cholesterol (VLDL-C)], aortic tissue MDA, and antioxidant reserve. Gross and microscopic changes in the aorta were also investigated. Because the TC/HDL-C and LDL-C/HDL-C ratios determine the relative risk of coronary artery disease, they were also calculated.

Methods

New Zealand White rabbits 6 to 8 weeks old weighing between 1.8 and 2.0 kg, after 1 week of adaptation, were assigned to 4 groups as shown in Table 1. Those in group 1 were fed rabbit laboratory chow pellets. The other groups received SDG or cholesterol or cholesterol plus SDG in addition to rabbit chow, as shown in Table 1. The diet was specially prepared by Purina and did not contain any antioxidants. SDG (15 mg/kg body wt) was fed orally, wrapped in leafy vegetables (lettuce). Water was given ad libitum. The rabbits were housed in cages under a 12-hour-light/12-hour-dark cycle according to approved standards for laboratory animal care. The rabbits were anesthetized at the end of 8 weeks, and aortas were removed under approved standards for laboratory animal care. The rabbits were housed in cages under a 12-hour-light/12-hour-dark cycle according to approved standards for laboratory animal care. The rabbits were anesthetized at the end of 8 weeks, and aortas were removed under pentobarbital sodium anesthesia (40 mg/kg IV) for measurement of serum TG, TC, HDL-C, LDL-C, and VLDL-C were collected before (time 0) and after 4 and 8 weeks on the respective experimental diets. No food was supplied for 18 hours before withdrawal of blood samples. Weights of the rabbits were recorded at 0, 4, and 8 weeks.

**TABLE 1. Experimental Diet Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n=8)</td>
<td>Control (laboratory rabbit chow)</td>
</tr>
<tr>
<td>2 (n=5)</td>
<td>SDG control (control diet supplemented with SDG 15 mg · kg body wt⁻¹ · d⁻¹ PO)</td>
</tr>
<tr>
<td>3 (n=6)</td>
<td>Cholesterol (1% cholesterol in rabbit chow diet)</td>
</tr>
<tr>
<td>4 (n=5)</td>
<td>Cholesterol+SDG (1% cholesterol diet supplemented with SDG 15 mg · kg body wt⁻¹ · d⁻¹ PO)</td>
</tr>
</tbody>
</table>

**Serum TG and Cholesterol**

As previously described, an automated chemistry analyzer (Hitachi model 717, Boehringer Mannheim) was used to measure serum TG, TC, and HDL-C. VLDL-C was calculated as the concentration of TG divided by 2.2. Serum LDL-C was calculated by subtracting the sum of HDL-C and VLDL-C from total cholesterol.

**Preparation of Aortic Tissue Homogenate and Supernatant**

Aortas (between the origin and bifurcation of iliac arteries) were removed, cleaned of gross adventitial tissue, and divided longitudinally into 2 halves. One half was used for estimation of atherosclerotic plaques and histology. The other half was used to prepare homogenate and supernatant by a previously described method.

**MDA (Thiobarbituric Acid–Reactive Substances)**

MDA levels in the homogenate were measured as thiobarbituric acid–reactive substances as previously described. Thiobarbituric acid–reactive substances were extracted in a mixture of butanol and pyridine, which was separated by centrifugation. The fluorescence intensity of the butanol/pyridine solution was measured at 553 nm with excitation at 513 nm. The MDA content of the aortic tissue was expressed as nmol/mg protein.

**Aortic Tissue Chemiluminescence**

Aortic tissue chemiluminescence (AO-CL), a measure of antioxidant reserve, was measured as previously described. An increase in tissue CL indicates a decrease in antioxidant reserve of tissue and vice versa. Antioxidant reserve is the amount of antioxidant present in the tissue at the time of exposure of the tissue to oxidants. Aortic tissue supernatant (0.8 mL) was added to a counting vial containing 0.4 mL of 2 × 10⁻² mol/L luminol and placed in a luminometer at 37°C. Reaction was initiated by adding 0.2 mL of 2 × 10⁻³ mol/L tert-butyl hydroperoxide (t-BHP). The CL for each sample was determined with or without t-BHP. The difference in the areas with and without t-BHP was designated as luminol-amplified CL (t-BHP–derived oxygen radicals). The CL was expressed as mV · s⁻¹ · mg protein⁻¹.

**Assessment of Atherosclerotic Plaques**

The atherosclerotic plaques were assessed with Herxheimer’s solution as previously described. The surface area of atheromatous lesions was measured from a photograph of the aorta and expressed as a percentage of total aortic intimal surface area. Small portions of the plaques and adjacent normal aortic area from groups 3 and 4 and from comparable areas of aorta from groups 1 and 2 were cut across and embedded in paraffin. Paraffin sections of aorta were cut and stained with hematoxylin–oil red O for lipid deposit and Verhoeff–van Gieson’s stain for elastic fibers and for morphological assessment of atherosclerotic lesions.

**Statistical Analysis**

Results are expressed as mean±SEM. Repeated-measures ANOVA was used for statistical analysis of blood lipid data and body weight. The Kruskal-Wallis test was used to test the differences in the atherosclerotic change in the 4 groups. The Mann-Whitney U test was used to determine the significance of differences between any 2 groups. Type I error for multiple comparison was controlled by Bonferroni correction. A value of P<0.05 was considered significant.

**Results**

**Body Weight**

The changes in the body weights of the rabbits of the experimental groups are shown in Table 2. There was a
progressive increase in the body weight of all groups. The weight gains in groups 2, 3, and 4 were similar but lower than in group 1.

### Triglycerides

Initial values for serum TG were similar in all groups except group 4, in which the values were lower than in group 3. Changes in TG level in the 4 groups are summarized in Figure 2. TG decreased in groups 1 and 2 at weeks 4 and 8 compared with time 0 but remained practically unchanged in the other groups. Values in groups 1 and 2 were lower than those in groups 3 and 4 at weeks 4 and 8.

### Cholesterol and Lipoproteins

Initial values for serum cholesterol in groups 1, 2, 3, and 4 were $2.26 \pm 0.20$, $1.42 \pm 0.11$, $2.68 \pm 0.20$, and $2.26 \pm 0.25$ mmol/L, respectively, and were not significantly different from each other except for that in group 2, which was lower than in the other groups. The results are summarized in Figure 3. Values remained unchanged in groups 1 and 2 at weeks 4 and 8 but increased markedly in groups 3 and 4, those in group 4 at week 8 being lower than those in group 3. Initial values for serum LDL-C in groups 1, 2, 3, and 4 were $0.90 \pm 0.10$, $0.34 \pm 0.09$, $1.22 \pm 0.17$, and $1.09 \pm 0.19$ (SEM) mmol/L, respectively. The results are summarized in Figure 4. Values remained unchanged in groups 1 and 2 compared with time 0 but increased in groups 3 and 4 at weeks 4 and 8, the increase being greater in group 3 than in group 4 at week 8.
Initial values for serum HDL-C were similar in all groups. The changes in values of serum HDL-C are summarized in Figure 5. Values for HDL-C remained unchanged in groups 1 and 2 throughout the period of observation compared with time 0 but decreased in group 3 and increased in group 4 at week 4 only.

The changes in the values for VLDL-C are shown in Figure 6. Initial values were similar in all groups except group 3, in which it was higher than in groups 1 and 4. It decreased in groups 1 and 2 at weeks 4 and 8 and group 3 at week 8 but remained unchanged in group 4 compared with time 0.

Initial values of TC/HDL-C ratio were 3.27 ± 0.21, 2.55 ± 0.20, 3.81 ± 0.27, and 3.74 ± 0.24 in groups 1, 2, 3, and 4, respectively, and were not significantly different from each other. Initial values of the LDL-C/HDL-C ratio in groups 1, 2, 3, and 4 were 1.31 ± 0.112, 0.570 ± 0.155, 1.73 ± 0.225, and 1.75 ± 0.322, respectively. Values in groups 3 and 4 were higher and in group 2 were lower than in group 1. Changes in the TC/HDL-C and LDL-C/HDL-C ratios are summarized in Figure 7. These ratios remained unchanged throughout the period of observation in groups 1 and 2 but increased in groups 3 and 4 compared with time 0. The increases in group 4 were smaller than those in group 3.

Aortic Tissue MDA
The MDA content of aortic tissue from the 4 groups is summarized in Figure 8A. It was 0.09 ± 0.01 nmol/mg protein in group 1. The level was lower in group 2, but not significantly so, and higher in groups 3 and 4 than in group 1. The levels of MDA were lower in group 4 than in group 3.

Aortic Tissue CL
The AO-CL in the 4 groups is summarized in Figure 8B. The value for chemiluminescent activity in group 1 was 8058 ± 619 (SEM) mV · s⁻¹ · mg protein⁻¹. It increased in group 3 compared with group 1 or 2 but decreased in group 4 compared with group 3.

Atherosclerotic Changes in Aorta
Representative photographs of endothelial surfaces of aortas from each group are shown in Figure 9, and the results are
summarized in Figure 10. Atherosclerotic plaques were absent in groups 1 and 2. However, a significant area of aortic intimal surface from group 3 (78.97 ± 5.44%) and group 4 (21.69 ± 2.06%) was covered with atherosclerotic plaques. In group 4, the atherosclerotic plaques were significantly smaller than those in group 3. SDG reduced the hypercholesterolemic atherosclerosis by 73%. Atherosclerotic plaques were distributed all over the aorta in group 3 but were present mainly in the proximal segment of the aorta and at the opening of the vasa vasorum in group 4.

Histological sections of aorta stained with hematoxylin–oil red O and Verhoeff–van Gieson’s stain from the 4 groups are shown in Figures 11 and 12, respectively. Histological sections through the atherosclerotic plaques of aortas from groups 3 and 4 showed thickening of the intima, which consisted of foam cells that contained oil red O–stainable lipid (Figure 11). The internal elastic lamina and elastic fibers in the subintimal media were intact and arranged in normal fashion (Figure 12). The overall thickness of the media increased in groups 3 and 4 compared with groups 1 and 2, being greater in group 3 than in group 4. The histological changes in groups 3 and 4 were qualitatively similar.

**Discussion**

The lower weight gains in groups 2, 3, and 4 compared with group 1 cannot be explained at present. It could not be due to SDG, because group 3, which did not have SDG in the diet, had a weight gain similar to that of group 4, which had SDG in the diet.

A high-cholesterol diet produced an increase in serum TC, LDL-C, and TC/HDL-C and LDL-C/HDL-C ratios and a decrease in HDL-C. Serum TG and VLDL-C remained unaffected. Qualitatively similar changes have been reported in earlier studies.1,2,25 In the present study, SDG reduced the levels of serum cholesterol and LDL-C and the TC/HDL-C and LDL-C/HDL-C ratios but increased the levels of HDL-C as early as 4 weeks in cholesterol-fed rabbits. The mechanism of these changes with SDG is not known. It should be noted that we have shown flaxseed to increase serum cholesterol, LDL-C, and VLDL-C in a high-cholesterol diet.14

The aortic MDA increased in rabbits fed the high-cholesterol diet but was decreased with SDG treatment. We have previously reported increases in aortic MDA in hypercholesterolemic rabbits.1,2 No data are available in the literature for

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Aortic tissue MDA (A) and CL (B) in 4 groups. Results are expressed as mean ± SEM. *P < 0.05, group 1 vs other groups. †P < 0.05, group 2 vs groups 3 and 4. ‡P < 0.05, group 3 vs group 4.

![Figure 9](https://example.com/figure9.png)

**Figure 9.** Intimal surface of aorta from 4 experimental groups showing Sudan IV–stainable lipid deposits. Note marked bright red lipid deposits in groups 3 and 4.
the effect of SDG on the level of aortic tissue MDA in hypercholesterolemic or normocholesterolemic rabbits. The decrease in MDA levels could be due to the antioxidant activity of SDG.18

An increase of AO-CL in rabbits fed a high-cholesterol diet suggests a decrease in antioxidant reserves, whereas a decrease in AO-CL with SDG treatment suggests an increase in the levels of antioxidant reserve. The increase in antioxidant reserve with SDG could be due to a reduction in oxidative stress because of the antioxidant activity of SDG.18 An increase in the antioxidant reserve of aortic tissue in hypercholesterolemic rabbits has been observed with antioxidants such as probucol2 and purpurrogallin,25

The severity of the atheromatous lesions in aorta was associated with hypercholesterolemia. We and other investigators1,2,26,27 have made similar observations. Hypercholesterolemic diet produced intimal thickening that contained foam cells similar to that observed by others.1,2,27 SDG reduced the extent of development of atherosclerosis in hypercholesterolemic rabbits. The protective effect of SDG could be attributed to its antioxidant activity, because it reduced levels of MDA and increased the antioxidant reserve in aortic tissue. SDG-induced decreases in serum TC and LDL-C and increases in HDL-C in hypercholesterolemic rabbits could also contribute to a reduction in atherosclerosis.

Hypercholesterolemic atherosclerosis was associated with an increase in aortic tissue MDA and a decrease in the antioxidant reserve. Increased aortic tissue MDA suggests an increase in the levels of OFRs, which may be due to increased production and/or decreased antioxidant reserve. Increased levels of OFRs may be due to increased production by PMNLs,1 endothelial cells, and other blood-borne or vessel-wall cells28 and during prostaglandin29 and leukotriene30 synthesis. A decrease in antioxidant reserve may also lead to increased levels of OFRs, which are known to produce endothelial cell injury.31 Endothelial cell injury represents a critical initiating event in the development of atherosclerosis.32 SDG may have prevented OFR-induced endothelial-cell injury through its antioxidant activity. Further studies with various dosages of SDG and for long durations are planned. This compound needs to be tested in human beings after animal studies are completed.
In conclusion, these results suggest that hypercholesterolemic atherosclerosis is associated with an increase in oxidative stress in aorta and that SDG is effective in reducing hypercholesterolemic atherosclerosis by reducing oxidative stress and lowering serum levels of cholesterol and LDL-C and raising serum levels of HDL-C in the early stage. SDG therefore may be useful in preventing hypercholesterolemic atherosclerosis and lowering the relative risk of coronary artery disease.

Acknowledgments
This work was supported by the Heart and Stroke Foundation of Saskatchewan. The author acknowledges the technical assistance of P.K. Chattopadhyay and P. Lee. The author is also grateful to Drs A.D. Muir and N.D. Westcott of Agriculture and Agri-Food Canada (AAFC), Saskatoon, for providing SDG.

References


Reduction of Serum Cholesterol and Hypercholesterolemic Atherosclerosis in Rabbits by Secoisolariciresinol Diglucoside Isolated From Flaxseed

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Circulation. 1999;99:1355-1362
doi: 10.1161/01.CIR.99.10.1355

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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